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A validated high-performance liquid chromatography method for detecting geniposide, ellagic acid, piperine, costunolide and dehydrocostus lactone in Liuwei Muxiang capsules

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Abstract: In this study, a sensitive high-performance liquid chromatography detector was established and validated for the simultaneous determination of geniposide, ellagic acid, piperine, costunolide and dehydrocostuslactone in Liuwei Muxiang Capsules. The analysis was achieved on CHANIN 100-5-C18-H column (5μm, 250 mm×4.6 mm) with the temperature of 30°C. Gradient elution was applied using 0.1% phosphoric acid solution-methanol-acetonitrile (50:50) as mobile phase at the flow rate of 1.0 mL/min. The determination was performed at the wavelength of 225 nm (detecting geniposide), 254 nm (detecting ellagic acid), 343 nm (detecting piperine) and 225 nm (detecting costunolide and dehydrocostuslactone) along with the sample volume of 10μL. The linear ranges of geniposide, ellagic acid, piperine, costunolide and dehydrocostuslactone demonstrated good linear relationships within their respective determination ranges. The average recoveries were 100.04%, 99.86%, 99.79%, 100.17% and 100.41%, respectively. RSD% was 1.3%, 1.2%, 1.2%, 1.2%, 1.5%, respectively. The developed method was proved to be simple, accurate and sensitive, which can provide a quantitative analysis method for the content determination of geniposide, ellagic acid, piperine, costunolide and dehydrocostuslactone in Liuwei Muxiang capsules.

Keywords: Geniposide, ellagic acid, piperine, costunolide, dehydrocostuslactone, Chinese medicine.

INTRODUCTION

Liuwei Muxiang Capsules (LMC) is composed of wood incense, gardenia, pomegranate peel, noisy sheep flower, cardamom and piper longum. It has the effect of relieving depression and relieving pain. Clinical treatment is mainly for stomachache, abdominal pain, belching and vomiting. In the current standard, only the content of geniposide in gardenia jasminoides was determined, but the main components of other traditional Chinese medicines were not involved. The existing literature (Hai *et al*., 2014; Ying *et al*., 2007; Yu *et al*., 2019; Ouni *et al*., 2021; Balkrishna *et al*., 2021) only focuses on the quantitative analysis of 1~2 components in the drug, which covers the limited medicinal materials, which is not conducive to the overall quality control of the drug. After literature research, according to the principles drawn up according to the quality standard, because ellagic acid is the effective ingredient of pomegranate skin, piperine is the effective ingredient of Piper longum and costunolide and dehydrocostuslactone are the effective components of Radix Aucklandiae. In order to better control the internal quality of LMC, HPLC method was used to increase the quantitative determination of ellagic acid, piperine, costunolide and dehydrocostus lactone. At the same time, the contents of geniposide, ellagic acid, piperine, costunolide and dehydrocostuslactone were determined to further improve the controllability of the quality of the drug. The chemical structure of these five constituents is displayed in fig. 1 (Shityakov *et al*., 2019; Vattem *et al*., 2005).

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MATERIALS AND METHODS

Instruments and materials

The quantitative analysis was accomplished on Agilent 1260 HPLC system. Electronic balance (XP26) was obtained from Mettler Toledo International Co., Ltd. Ultrasonic cleaner (KQ5200) was purchased from Kunshan Ultrasonic Instrument Co., Ltd.

The reference substance of geniposide (batch No. 110749-201919), costunolide (batch No. 111524-201710) and dehydrolignolactone (batch No. 111525-201711) were obtained from China Institute for Food and Drug Control. The reference substance of ellagic acid (batch No. MUST-22042202) and piperine (batch No. MUST-21080806) was purchased from Chengdu Manster Biotechnology Co., Ltd. Water was purified through 0.22 μm filter membrane, methanol and acetonitrile were chromatographic pure, and other reagents were analytical alcohols. LMC (Jiangxi Derui Pharmaceutical Co., Ltd., batch No. 20210502). Flos Rhododendri Mollis (Hubei Rich Agriculture, batch No.20200103). Cardamom

(Hangzhou East China Traditional Chinese Medicine Co., Ltd., batch No.191231).

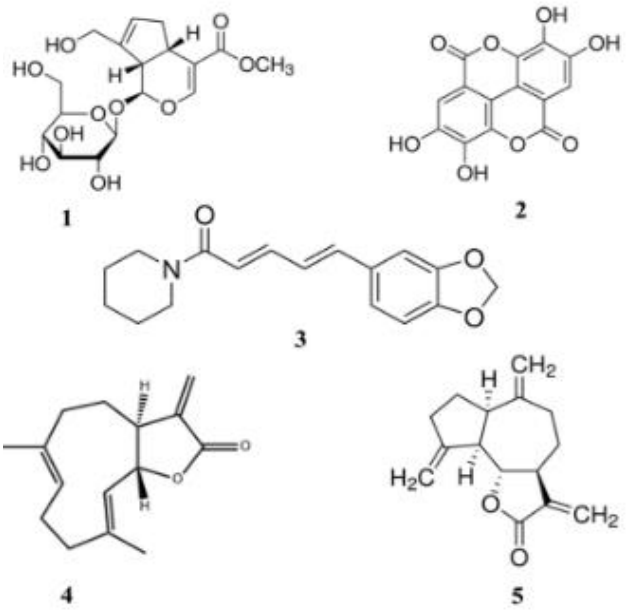

Fig. 1: Chemical structure of five components. (1. Geniposide; 2. ellagic acid; 3. piperine; 4. costunolide; 5. dehydrocostuslactone)

Chromatographic conditions

The simultaneous determination was performed on CHANIN 100-5-C18-H column (5μm, 250mm× 4.6mm, Gunning science and technology). The mobile phase was composed of 0.1% phosphoric acid solution (A) and methanol-acetonitrile (50:50) (B). The detailed gradient elution conditions were as below: 0~8 min, 90%A; 8~15min, 90%→60%A; 15~20min, 60%A; 20~25min, 60%→25%A; 25~40min, 25% A. The detective wavelengths were as below: 0~18 min, 238 nm (detecting geniposide), 18~25 min, 254 nm (detecting ellagic acid), 25~32 min, 343 nm (detecting piperine) and 32~40 min, 225 nm (detecting costunolide and dehydrocostuslactone). The column temperature was maintained at 30□. The flow rate was set as 1.0mL/min. The sample volume was 10μL (Wu *et al*., 2020).

Preparation of standard solutions

The geniposide reference substance was weighed at 10.47 mg (97.1% purity), the piperine reference substance at 12.83 mg (98.45% purity), the costunolide at 13.83 mg (99.5% purity) and the dehydrocostuslactone reference substance at 13.00mg (99.8% purity). Each substance was then dissolved and diluted with methanol in a 25mL bottle, thoroughly shaken and stored as a reference substance. Accurately measured the ellagic acid reference substance, weighed 12.03mg (99.84%), and transfered it to a 25mL measuring vial. Added DMSO and added ultrasonic to dissolve the substance. Diluted the solution to the desired scale, shake it thoroughly and kept it as the reference substance.

Mixed reference solution: 2.8mL of geniposide, 1.0mL of ellagic acid, 1.3mL of piperine, 1.8mL of costunolide and 1.3ml of dehydrolignolactone were precisely removed and placed in 10mL flask, dissolved and diluted to scale with methanol, shaken well.

Sample extraction duration investigation

During the test solution preparation, the ultrasonic extraction time (Rodsamran *et al*., 2019) was compared by primarily analyzing three time points: 30, 60 and 90 minutes. The variations in peak area of the five active components were utilized to ascertain their full extraction.

Preparation of test solutions

10 tablets of LMC were taken and their contents were carefully poured out. The contents were then accurately weighed, mixed and ground. From this mixture, 1.0g was precisely weighed and placed in a volumetric bottle. 50mL of methanol was added to the bottle, which was then sealed and weighed. The bottle was subjected to ultrasonic treatment for 60 minutes and weighed again. The lost weight was compensated for by adding methanol. The mixture was thoroughly shaken and filtered using a microporous membrane (0.45μm), resulting in a continuous filtrate.

Preparation of negative control solutions

The experiment involved taking 0.14 g of rhododendri mollis flos (Feng *et al*., 2021) and 0.10 g of cardamom, crushing them into a fine powder and adding 50mL of methanol. The mixture was then subjected to ultrasonic treatment for 60 minutes. After this, the weight was measured again and any lost weight was compensated by adding methanol. The mixture was then shaken well and filtered using a micro porous membrane with a pore size of 0.45μm, resulting in the filtrate.

Method validation

Validation experiments of the proposed method was carried out in this study, which included the following parameters: Specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, stability, accuracy.

The specificity of the HPLC analysis was determined by comparing the chromatograms obtained, in order to identify any potential interference from prescription factors. The reference substance solution, test solution, and negative control solution were introduced into the HPLC system and the resulting chromatograms were collected at various wavelengths.

Specialized results

The effect of the blank solvent on the major peaks of the five active components was examined by injecting a

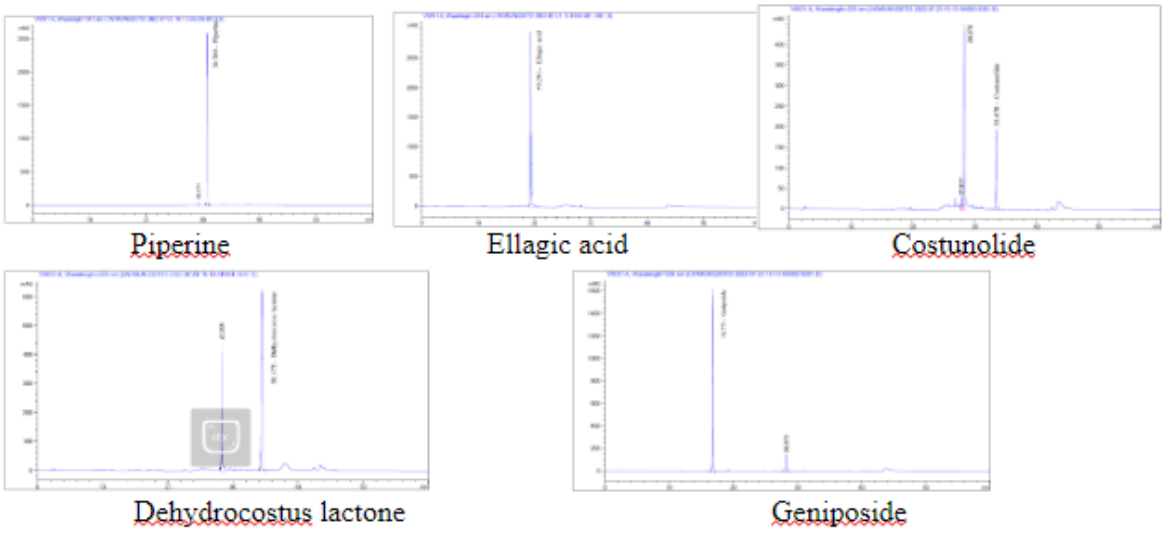

Fig. 2: Positioning chromatogram of five components.

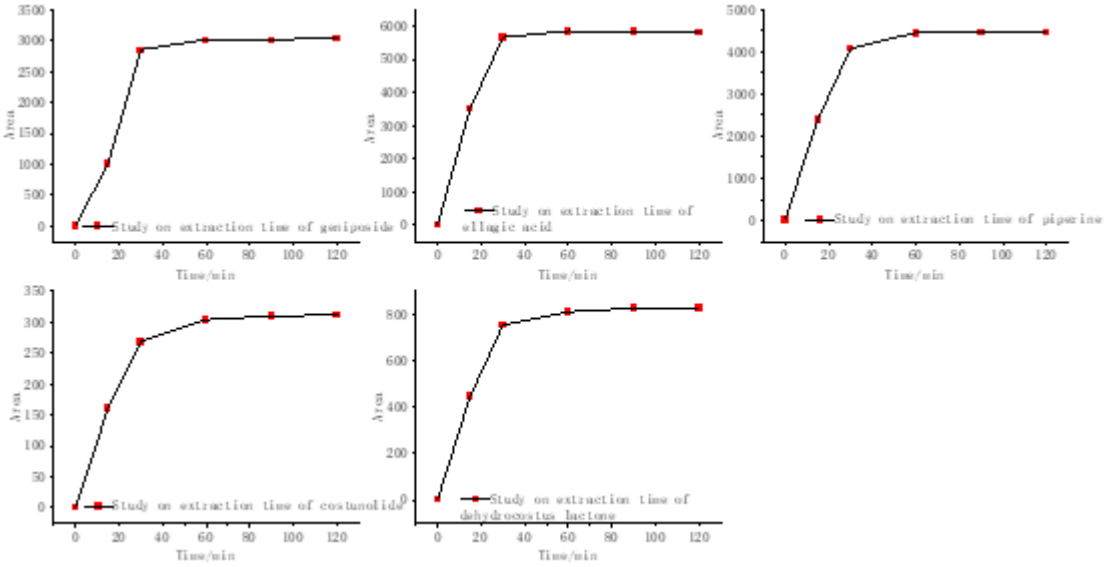

Fig. 3: Results of ultrasound extraction duration assessment (1. Geniposide; 2. ellagic acid; 3. piperine; 4. costunolide; 5. dehydrocostuslactone)

combined solution of reference material and blank solvent into the liquid phase. The chromatographic peaks and adjacent peaks must have a resolution greater than 1.5, and the theoretical number of plates should exceed 2000.

Linearity

The linearity was assessed using standard curves consisting of six data points. The geniposide, ellagic acid, piperine, costunolide and dehydrocostuslactone reference solutions were accurately extracted and mixed with methanol to provide a range of standard solutions. The geniposide reference solutions have concentrations of 22.77, 56.93, 91.09, 113.86, 136.63 and 227.72μg/mL. The ellagic acid reference solutions have concentrations of 9.6, 24, 38.4, 48, 57.6 and 96μg/mL. The piperine reference solutions have concentrations of 13.13, 32.82, 52.5, 65.63, 78.76 and 131.26μg/mL. The costunolide reference solutions have concentrations of 19.82, 49.54, 79.26, 99.08, 118.9 and 198.16μg/mL. The dehydrocostuslactone reference solutions have concentrations of 13.49, 33.73, 53.97, 67.46, 80.95 and 134.92μg/mL, respectively. Subsequently, the aforementioned set of solutions was introduced into HPLC for analysis. The LOD and LOQ were established based on the concentration or quantity that yielded a signal-to-noise ratio of 3:1 and 10:1, respectively (Xu *et al*., 2021).

Precision

The precision was assessed using HPLC analysis of the standard solution within a 24-hour period. The chromatographic peak area and relative standard deviation

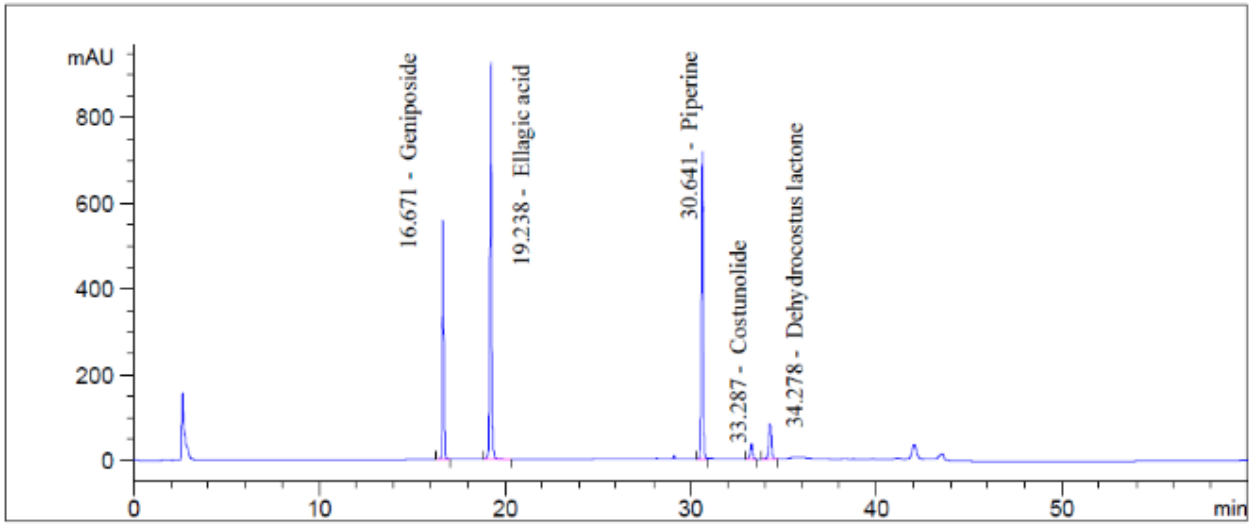

Fig. 4: Reference standard of five components. (1. Geniposide; 2. ellagic acid; 3. piperine; 4. costunolide; 5. dehydrocostuslactone)

(RSD%) of each component were recorded and computed based on the chromatographic circumstances. This was done by injecting the reference solution (100% linearity solution) six times consecutively. The method's repeatability was assessed by measuring five components in six distinct test solutions. Six aliquots from the identical batch of LMC (batch No. 20210502) were prepared for HPLC examination. Furthermore, the RSD% were estimated individually.

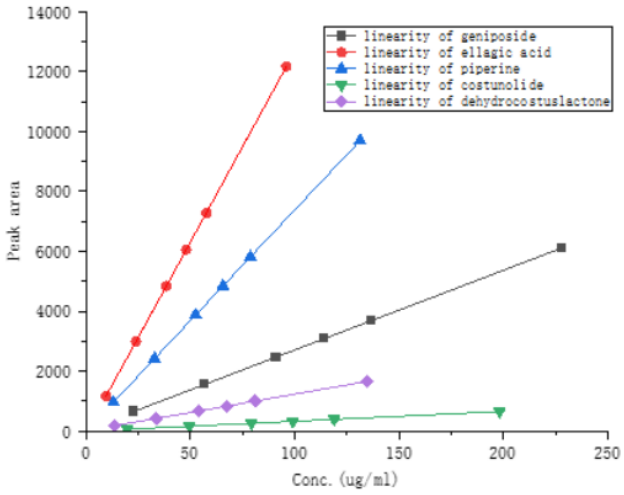

Fig. 5: Linearity of five components. (1. Geniposide; 2. ellagic acid; 3. piperine; 4. costunolide; 5. dehydrocostuslactone)

Accuracy

The accuracy of the method was evaluated by recovery experiment. Nine LMC samples (0.5g) with known content were accurately weighed and put into 9 different conical bottles with plugs. The standard solutions were added to the 9 conical bottles to make the test solution. Finally, the test solutions were injected into the chromatographic system for detection.

Stability

The stability was investigated by analyzing the peak area changes of five components within 12 hours. The same test solution was injected into the chromatographic system at 0,2,4,8 and 12 hours for determination. The RSD% of the peak area of five components in the test solution at different time points were calculated.

Determination of active components

Three batches of LMC samples were taken and made into test solution, respectively. The above solution was filtered through a microporous filter (0.22μm) and the continued filtrate were determined by HPLC. In addition, peak area was recorded and the contents of five different components were calculated by external standard method.

RESULTS

Inspection results of detection wavelength and extraction time for 5 active ingredients

As shown in fig. 2, the mixed reference solution was scanned at 190~400nm and the maximum absorption wavelength of each component was obtained. According to the Pharmacopoeia of the People's Republic of China and related literature (Fei *et al.*, 2019; Fu *et al.*, 2020), the detection wavelengths were determined as 238 nm (detection of geniposide), 254 nm (detection of ellagic acid), 343 nm (detection of piperine) and 225 nm (detection of costunolide and dehydroandrographolide).

The study examined various extraction durations (30 minutes, 60 minutes and 90 minutes), as depicted in fig. 3.

Table1: Linear relationships of various constituents.

Constituent	Standard curve	Linearity range /μg·mL^{-1}	r^2	LOD /μg·mL^{-1}	LOQ /μg·mL^{-1}
Geniposide	Y=26.634X+44.925	22.77~227.72	0.9997	0.35	1.16
Ellagic acid	Y=127.47X-63.063	9.60~96.00	0.9995	0.63	2.10
Piperine	Y=73.976X-11.343	13.13~131.26	0.9999	1.03	3.43
Costunolide	Y=3.3134X-2.0147	19.82~198.16	0.9998	1.35	4.50
Dehydrocostuslactone	Y=12.226x+11.331	13.49~134.92	0.9998	0.41	1.37

Table 2: Precision results of various constituents

Constituent	Peak area of 100% linear solution	RSD %
Geniposide	3077.472	0.2
	3067.128	
	3078.570	
	3077.139	
	3079.254	
	3077.580	
Ellagic acid	6055.497	0.3
	6056.562	
	6059.581	
	6050.711	
	6049.781	
	6015.581	
Piperine	4843.702	0.3
	4840.601	
	4820.981	
	4811.596	
	4850.666	
	4823.123	
Costunolide	326.280	1.1
	325.348	
	330.45	
	329.879	
	335.066	
	325.891	
Dehydrocostus lactone	799.428	1.7
	780.468	
	789.882	
	779.251	
	784.630	
	760.125	

The results indicated that following a 60 minutes extraction, all five components in the sample were effectively removed in their entirety. The test sample underwent ultrasonic extraction for a duration of 60 minutes, taking into account the extraction rate and economic variables.

Specificity results

The chromatogram acquired from the specificity assessment, as depicted in fig 4. The fig. demonstrates that there was no interference peak observed at the specific site of each analyte when detected at multiple wavelengths. Furthermore, the measured chromatographic peak had a resolution greater than 1.5 when compared to the adjacent peak, demonstrating a high level of specificity in the approach.

Linear results

Fig. 5 illustrated the establishment of the calibration curve using the standard solution concentration (X) as the horizontal axis and the chromatographic peak area (Y) as the vertical axis. Furthermore, the LOD and the LOQ were computed. The computation outcomes were displayed in table 1.

Table 3: Repeatability results of various constituents.

Constituent	Content% (sample batch No. 20210502)	RSD %
Geniposide	5.31	1.1
	5.29	
	5.20	
	5.29	
	5.22	
	5.35	
Ellagic acid	2.79	1.5
	2.75	
	2.68	
	2.78	
	2.71	
	2.75	
Piperine	2.94	1.3
	2.91	
	2.95	
	2.89	
	2.99	
	2.90	
Costunolide	4.55	0.3
	4.54	
	4.53	
	4.55	
	4.56	
	4.57	
Dehydrocostus lactone	3.23	1.2
	3.25	
	3.20	
	3.21	
	3.30	
	3.20	

The findings indicated that the peak area of geniposide, ellagic acid, piperine, costunolide and dehydrocostuslactone exhibited a strong linear correlation within their respective linear ranges. This suggested that the method used in this study had high sensitivity (Zhang *et al*., 2021).

Precision results

As shown in table 2 and table 3, the RSD% (n=6) of the peak area of geniposide, ellagic acid, piperine, costunolide and dehydrocostus lactone were 0.2%, 0.3%, 0.3%, 1.1%, 1.7%, respectively. The RSD% were all less than 2.00%, indicating that the method had high precision. In the repeatability test, the RSD% (n=6) of contents of the five components in the test sample (batch No. 20210502) were 1.5%, 1.5%, 1.3%, 0.3%, 1.2%, respectively and the results showed that the repeatability was good.

Accuracy results

The recovery rates of the five active components, as indicated in table 4, were 100.04%, 99.86%, 99.79%, 100.17% and 100.41% correspondingly. All the recovery rate results satisfied the specified criteria and the relative standard deviation (RSD %) of the recovery rate was below 2.0. The validation results of the recovery rate were satisfactory.

Stability results

The stability test yielded RSD% of 1.1%, 0.4%, 1.3%, 0.7% and 1.0% for the peak areas of geniposide, ellagic acid, piperine, costunolide and dehydrocostus lactone, respectively. The test was conducted with a sample size of 6 (n=6). All of the RSD % values were below 2.00%, which suggests that the tested solution was stable for a duration of 12 hours.

The presence of five constituents was found and the recovery rates (as shown in table 2) were determined for each one. The average recoveries of the five test samples varied from 99.86% to 100.41% with RSD% ranging from 1.2% to 1.5%. The aforementioned results indicate that the analytical method exhibited a high level of accuracy (Zhang *et al*., 2022; Fu *et al*., 2020).

Simultaneous determination of five active components in LMC

An HPLC approach was developed to simultaneously determine five active components in samples. The findings were displayed in table 5. The levels of geniposide and costunolide in the analysed samples exceeded those of the other components. The concentrations of five active constituents in the same batch of LMC exhibited a high degree of stability, with RSD % of less than 1.65%.

The composition of different batches of samples exhibited variations, with RSD% exceeding 2.3%. This discrepancy could perhaps be attributed to disparities in the quality of traditional Chinese medicine. Hence, it was imperative to implement stringent quality control measures for traditional Chinese medicine in order to guarantee the quality of compound formulations.

DISCUSSION

This study established a sensitive high-performance liquid chromatography method for the simultaneous determination of geniposide, ellagic acid, piperine, costunolide and dehydrocostus lactone in Liuwei Muxiang capsules and conducted complete methodological validation of the method. The linear ranged of five active ingredients showed good linear relationships within their respective measurement ranges, with linear correlation coefficients r^2 greater than 0.999. Blank excipients have no interference with the main components and the separation degree is greater than 1.5, indicating good specificity.

Table 4: Results of recovery test of five active constituents (n=9).

Constituent	Known content mg	Added amount mg	Measured amout mg	Recovery %	Average recovery %	RSD %
Geniposide	2.67	2.57	5.21	99.50	100.04	1.3
	2.71	2.57	5.32	100.67		
	2.66	2.57	5.35	102.37		
	2.71	2.57	5.27	99.76		
	2.67	2.57	5.15	98.25		
	2.70	2.57	5.22	99.13		
	2.67	2.57	5.29	100.87		
	2.68	2.57	5.18	98.65		
	2.71	2.57	5.34	101.20		
Ellagic acid	1.40	1.42	2.79	98.91	99.86	1.2
	1.43	1.42	2.87	100.83		
	1.40	1.42	2.77	98.38		
	1.43	1.42	2.84	99.81		
	1.40	1.42	2.81	99.51		
	1.42	1.42	2.89	101.89		
	1.41	1.42	2.85	100.88		
	1.41	1.42	2.79	98.63		
	1.42	1.42	2.84	99.93		
Piperine	1.48	1.52	2.97	99.13	99.79	1.2
	1.50	1.52	2.98	98.58		
	1.47	1.52	3.01	100.65		
	1.50	1.52	3.06	101.26		
	1.48	1.52	2.95	98.35		
	1.49	1.52	3.04	100.91		
	1.48	1.52	2.99	99.65		
	1.48	1.52	3.04	101.18		
	1.50	1.52	2.97	98.39		
Costunolide	2.28	2.35	4.60	99.25	100.17	1.2
	2.33	2.35	4.65	99.44		
	2.28	2.35	4.70	101.60		
	2.32	2.35	4.66	99.69		
	2.29	2.35	4.62	99.58		
	2.31	2.35	4.59	98.50		
	2.29	2.35	4.70	101.26		
	2.30	2.35	4.73	101.78		
	2.32	2.35	4.69	100.45		
Dehydrocostuslactone	1.62	1.67	3.35	101.77	100.41	1.5
	1.65	1.67	3.29	99.06		
	1.62	1.67	3.35	101.96		
	1.65	1.67	3.28	98.79		
	1.63	1.67	3.34	101.36		
	1.64	1.67	3.26	98.49		
	1.63	1.67	3.37	102.23		
	1.63	1.67	3.28	99.37		
	1.65	1.67	3.34	100.71		

Table 5: Results of content determination of various constituents (n=3).

Batch number	20210502		20190502		20191003		Batch RSD%
	Content	RSD %	Content	RSD %	Content	RSD %	
Geniposide	5.31	1.02	5.24	1.22	5.63	0.87	3.9
Ellagic acid	2.79	1.23	2.85	0.84	2.72	0.69	2.3
Piperine	2.94	0.74	2.78	0.68	2.72	1.65	4.0
Costunolide	4.55	0.84	4.32	1.30	4.51	1.11	2.8
Dehydrocostuslactone	3.23	0.51	3.28	0.33	3.55	0.54	5.1

In accuracy validation, the average recovery rates of the method were 100.04%, 99.86%, 99.79%, 100.17% and 100.41%, respectively, with RSD% of 1.3%, 1.2%, 1.2% and 1.5%. The RSD% of the six samples in precision were all less than 2.0. This method has the characteristics of simplicity, precision, accuracy and sensitivity. At present, in addition to the current standards that specify the content of geniposide, there is a small amount of literature on the active ingredients of the main drug - Muxiang in Liuwei Muxiang capsules. However, there is little research on the quality control of other components in Liuwei Muxiang capsules, and there are no reports on the simultaneous determination of geniposide, ellagic acid, piperine, costunolide, and dehydrocostuslactone content using HPLC, Therefore, the simultaneous determination of the content of the five components mentioned above using HPLC method developed in this article has important reference significance for the quality control of Liuwei Muxiang capsules. However, the detection time of the method developed in this article is as long as 60 minutes, which is not conducive to the inspection of large batches of samples in practical applications and will increase the response economic cost. Therefore, in response to the limitations of this method, we will further carry out relevant research, strive to shorten the detection time, improve efficiency, and provide readers with better quality methods.

CONCLUSION

The compound formulation of traditional Chinese medicine is identical to traditional Chinese medicine, exhibiting the attributes of intricate constituents and numerous bioactive compounds. The merger of traditional Chinese and Western medicine (Lee *et al*., 2020; Liu *et al*., 2020; Yang *et al*., 2020) has led to the need for quantifying active components, which is crucial for the development, application and quality control of medications. This paper presents the development of a straightforward, reliablec and fast HPLC method for the simultaneous quantification of five bioactive constituents (geniposide, ellagic acid, piperine, costunolide and dehydrocostus lactone) in LMC. The determination result exhibits the benefits of exceptional accuracy, reliable reproducibility, excellent recovery and straightforward analysis. This approach can establish a systematic framework and serve as a point of reference for ensuring the quality control of the preparation.

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